Universidade de Aveiro Departamento de Biologia

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Telma Luísa Machado Veloso

Diversity and antimicrobial potential of bacteria isolated from Algarve caves

Diversidade e potencial antimicrobiano de bactérias isoladas de grutas do Algarve

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Doutor Sérgio Miguel Reis Luís Marques, Investigador de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro e coorientação da Doutora Helena Cristina Correia de Oliveira, Investigadora de Pós-Doutoramento do Departamento de Biologia e do Professor Doutor Fernando Mendes Gonçalves, Professor Associado com Agregação do Departamento de Biologia.

Aos meus pais, Domingos e Fernanda.

À minha madrinha, Mónica.

o júri

Presidente

Doutor Artur Jorge da Costa Peixoto Alves
Investigador Principal com Agregação do Departamento de Biologia da Universidade de Aveiro

Doutora Verónica Isabel Correia Bastos
Investigadora em Pós-Doutoramento do Departamento de Ciências Químicas da Faculdade de Farmácia da Universidade do Porto

Doutor Sérgio Miguel Reis Luís Marques
Investigador de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro

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palavras-chave Grutas, diversidade, bactérias, atividade antimicrobiana

resumo

Recentemente, a necessidade de novas moléculas com atividade antimicrobiana aumentou devido ao contínuo aumento do número de bactérias patogénicas com resistência aos vários antibióticos disponíveis no mercado. Para colmatar esta necessidade, a comunidade científica tem realizado esforços na procura de novos microrganismos e dos seus compostos bioativos.

Grande parte do esforço tem sido concentrado em ambientes com condições extremas, uma vez que nestes ambientes os microrganismos que os habitam têm de ter características muito específicas. As grutas cársicas são consideradas ambientes extremos uma vez que a ausência de luz natural impede o crescimento de plantas, fazendo com que o ambiente seja caracterizado por elevada escassez nutritiva. Juntamente com a elevada humidade e a temperatura relativamente baixa e constante, faz com os microrganismos que habitam estes ambientes se tornem altamente especializados. O facto destes habitats únicos serem pouco estudados, nomeadamente no que diz respeito à comunidade bacteriana, torna-os num potencial reservatório, tanto de novas espécies como de novos compostos antimicrobianos, tal como demonstrado nos poucos estudos existentes. Considerando estes factos, os objetivos deste estudo foram: i) obter e identificar isolados bacterianos recolhidos em três grutas do Algarve nomeadamente, as grutas do Vale do Telheiro, da Senhora e do Ibne Ammar e ii) avaliar se os mesmo têm atividade antimicrobiana.

A partir das amostras recolhidas nas três grutas, foram isoladas 110 estirpes bacterianas. Após sequenciação e pesquisa de similaridades em bases de dados foi possível verificar que os isolados bacterianos pertencem a três filos, nomeadamente Firmicutes, Proteobacteria e Actinobacteria, num total de 19 géneros diferentes. Em relação à actividade antimicrobiana, os testes foram realizados contra seis agentes teste *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Aeromonas salmonicida* ATCC 33658 e *Bacillus cereus* ATCC 14579. Os resultados revelaram que aproximadamente 52% dos isolados apresentam atividade antimicrobiana contra, pelo menos, um agente teste e que, 10 isolados foram capazes de inibir o crescimento de mais de 4 agentes teste.

Este estudo foi o primeiro a focar a diversidade bacteriana cultivável de grutas do Algarve e confirmou que a prospeção de compostos antimicrobianos em ambientes subterrâneos poderá ser uma das estratégias para combater o problema da resistência aos antibióticos.

keywords

Caves, diversity, bacteria, antimicrobial activity.

abstract

Recently, the need for new molecules with antimicrobial activity increased due to a continuous the increasing number of multi drug resistant pathogenic bacteria. To fill this urging need, the scientific community has made a great investment in searching for new microorganisms and their bioactive compounds. A great part of the effort has been focused in environments with extreme conditions, since in these environments the inhabiting microorganisms must have very particular features. Karstic caves are considered extreme environments due to the nutrient scarcity caused by absence of natural light that precludes the growth of vascular green plants. In addition, the relatively low and stable temperature and the high humidity levels, force cave dwelling microorganisms to become highly specialized. The fact that these unique habitats are poorly studied, in particular when considering the bacterial community, makes them a potential reservoir for both new species and new antimicrobial compounds, as demonstrated in the few existing studies. Considering these facts our study aimed at: i) obtain and identify bacterial isolates sampled in three caves of Algarve, namely Vale do Telheiro, Senhora and Ibne Ammar and, ii) assess their antimicrobial activity.
From the collected samples of the three caves we are able to isolate 110 bacterial isolates. After sequencing and searching for homologies in the databases it was possible to include the isolates in phyla, namely Firmicutes, Proteobacteria and Actinobacteria, in a total of 19 genera. Regarding the antimicrobial activity, the tests were performed against six test agents: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Aeromonas salmonicida* ATCC 33658 and *Bacillus cereus* ATCC 14579. The results revealed that approximately 52% of the bacterial isolates presented antimicrobial activity against at least one test agent, and that ten bacterial isolates were able to inhibit the growth of more than four test agents.
This study was the first to focus cultivable bacterial diversity in the Algarve caves and confirmed that prospection for new antimicrobial compounds in subterranean environments might be one of the strategies for fighting the problem of multi drug resistant bacteria.

Index

I - General Introduction 1

1- Antibiotics 3

1.2- Classes, modes of action and examples 3

2- *Antibiotic resistance* 5

3- New antimicrobial compounds prospection 7

4- Caves as a suitable environment for prospecting microorganisms with novel antibiotic compounds 8

4.1- Caves 8

4.2- Cave bacteria 11

5- Goals and structure of the dissertation 15

II - Diversity and antimicrobial potential of bacteria isolated from Algarve caves 21

1- Introduction 23

2- Materials and Methods 25

2.1- Sampling site 25

2.2- Sampling, isolation and culture conditions 26

2.3- Amplification of 16S rDNA gene by PCR and sequencing 26

2.4- Screening test for antimicrobial activity 27

3- Results 28

3.1- Cultivable bacterial diversity 28

3.2- Antimicrobial activity 35

4- Discussion 38

5- References 41

III - Final Remarks 45

1- Final Remarks 47

2- References 49

I - General Introduction

1- Antibiotics

Antimicrobial compounds are chemical substances capable of kill or inhibiting the growth of microorganisms. The term is applied to antibacterial, antiviral, antifungal and antiparasitic agents. Normally, they are produced by plants, animals or microorganisms (Etebu and Arikekpar 2016). Also they are defined as low molecular weight organic natural products produced by microorganisms, such as secondary metabolites or idiolites, which are active against other microorganisms at low concentrations (Demain 2014).

Antimicrobial compounds are separated in three different categories: natural, semi-synthetic and synthetic. Natural compounds are originally extracted from plants, fungi, microorganisms and animals. Semi-synthetic compounds are natural compounds that have been chemicallly modified to minor toxicity effects or enlarge effectiveness rates. Synthetic compounds are totally chemically engineered and revealed to be more effective than natural or semi-synthetic agents (Etebu and Arikekpar 2016).

The antibiotic discovery remote to the beginning of 20th century with the discovery of arsphenamine (Salvarsan or compound 606) by Paul Ehrlich (Bosch and Rosich 2008). This drug has activity against the bacterium *Treponema pallidum*, which cause syphilis infection disease. In 1928 Alexander Fleming discovered Penicillin and, after many years of optimizing the producing process, this drug was used to treat many Gram-positive bacterial infections (Bosch and Rosich 2008). Some years later, Selman Waksman isolated streptomycin (Hathaway et al. 2014). The drug was applied to treat gram positive and negative bacteria and was the first treatment for tuberculosis (Bosch and Rosich 2008). Between 1945 and 1960, most of the antibiotics currently applied were discovered, being this period known as “Golden Era of Antibiotic Discovery”. Since then the discovery of new antibiotics was significantly slowed down and a new problem has emerged, the appearance of multi-drug resistant bacteria (Lewis 2017).

1.2- Classes, modes of action and examples

Antibiotics have several mechanisms of action on target cells. Their principal action modes depend on the target organelle and generally include inhibition of

vital cell processes such as protein synthesis, nucleic acid synthesis, cell wall synthesis and other metabolic pathways (Dowling, Dwyer, and Adley 2017).

The most relevant antibiotics produced by microorganisms include the β-lactams, tetracyclines, aminoglycosides, chloramphenicol, macrolides and glycopeptides (Etebu and Arikekpar 2016).

The class of β-lactam antibiotics is the oldest class of antibacterial agents and it includes penicillin and derivates, monobactms, carbapenems and cephalosporins (Etebu and Arikekpar 2016). All the members of β-lactam antibiotics contain a β-lactam ring in their molecular structure. These antibiotics irreversibly inhibit the activity of transpeptidase, an essential enzyme for cell wall synthesis.

Tetracyclines were discovered in the 1940s and are broad spectrum antimicrobial agents used to treat Gram positive and negative bacteria, mycoplasmas and protozoan parasite. They inhibit protein synthesis by interfering with amino acid transfer preventing the association of aminoacyl-tRNA with the bacterial ribosome (Chopra and Roberts 2001).

Aminoglycosides are a class of antibiotics that act on the inhibition of protein production, altering cell wall permeability and causing genetic code to misread by rRNA binding to 30S subunit. They are particularly important for treating Gram-negative infections, mainly caused by aerobic bacteria. Neomycin, streptomycin and kanamycin are examples of antibiotics included in this group and they were isolated from *Streptomyces* spp., *Micromonospora* spp. and *Bacillus* spp. (Demain 2014).

Chloramphenicol is bacteriostatic and was firstly isolated from *Streptomyces venezuelae* but today is produced artificially due to his simple structure (Demain 2014). It acts by inhibition of protein synthesis by blocking elongation of polypeptide chain.

Macrolides were first isolated in 1952 as a metabolic product of soil fungus *Saccharopolyspora erythraea*. They are often administrated to peniciin allergic patients and act by effectively inhibiting protein production. The compound binds to bacterial ribosome and block the addition of amino acids to polypeptide chain (Etebu and Arikekpar 2016).

Glycopeptides are the most relevant class of antibiotics applied to the treatment of aggressive infection caused by Gram-positive bacteria, such as

enterococci and MRSA- *Staphylococcus aureus*. Vancomycin is one of the antibiotics belonging to this group and basically it interferes with cell wall production in Gram-positive bacteria (Kang and Park 2015).

2- Antibiotic resistance

Antibiotic resistance is defined as the capacity of bacteria to resist the effects of an antibiotic to which they were previously sensitive, living and growing in his presence. This scenario is a consequence of more than 70 years of widespread use of antibiotics that allowed the evolution of pathogenic resistant bacteria and lead to the inefficacy of most of the available antibiotics being. From a clinical point of view an infection by an antibiotic resistant bacterium can present severe health problems, namely if the available antibiotics are no longer effective (Alnahdi 2014)

Drug resistance genes can be passed from one bacterium to another through several mechanisms (figure 1). Bacteria can acquire external DNA via transposons, plasmids, bacteriophages or naked DNA. Some transposons containing integrons can assemble different antibiotic genes or other gene cassettes, resulting in spreading of multiple resistance mechanism at once. Also, chromosomal genes can be transferred through a process named transformation: a bacterium collects naked DNA discharged by another bacterium (Levy and Bonnie 2004). Although resistant genes prevailed in clinical environments, scientists have discovered that resistance genes are a naturally occurring process which is encoded for by ancient microbial genes (Aminov 2010). Therefore, this natural process was amplified since the beginning of the Antibiotic Era. There are two components that cause drug resistance: the antimicrobial compound and the genetic resistance determinant (Levy 2002). The antimicrobial compound selects the bacteria that are resistant while the genetic resistance determinant in bacteria is selected by the antimicrobial compound (Levy and Bonnie 2004). When these two conditions are present in the environment or in a host, antimicrobial selection favours proliferation of resistant genes and host to another bacteria or geographic location (Levy 2002).

In our modern world millions of kilograms of antimicrobial agents are disseminated each year to treat people, animals and crops globally, and none of the classes of antibiotics escape a resistance mechanism (Levy and Bonnie 2004).

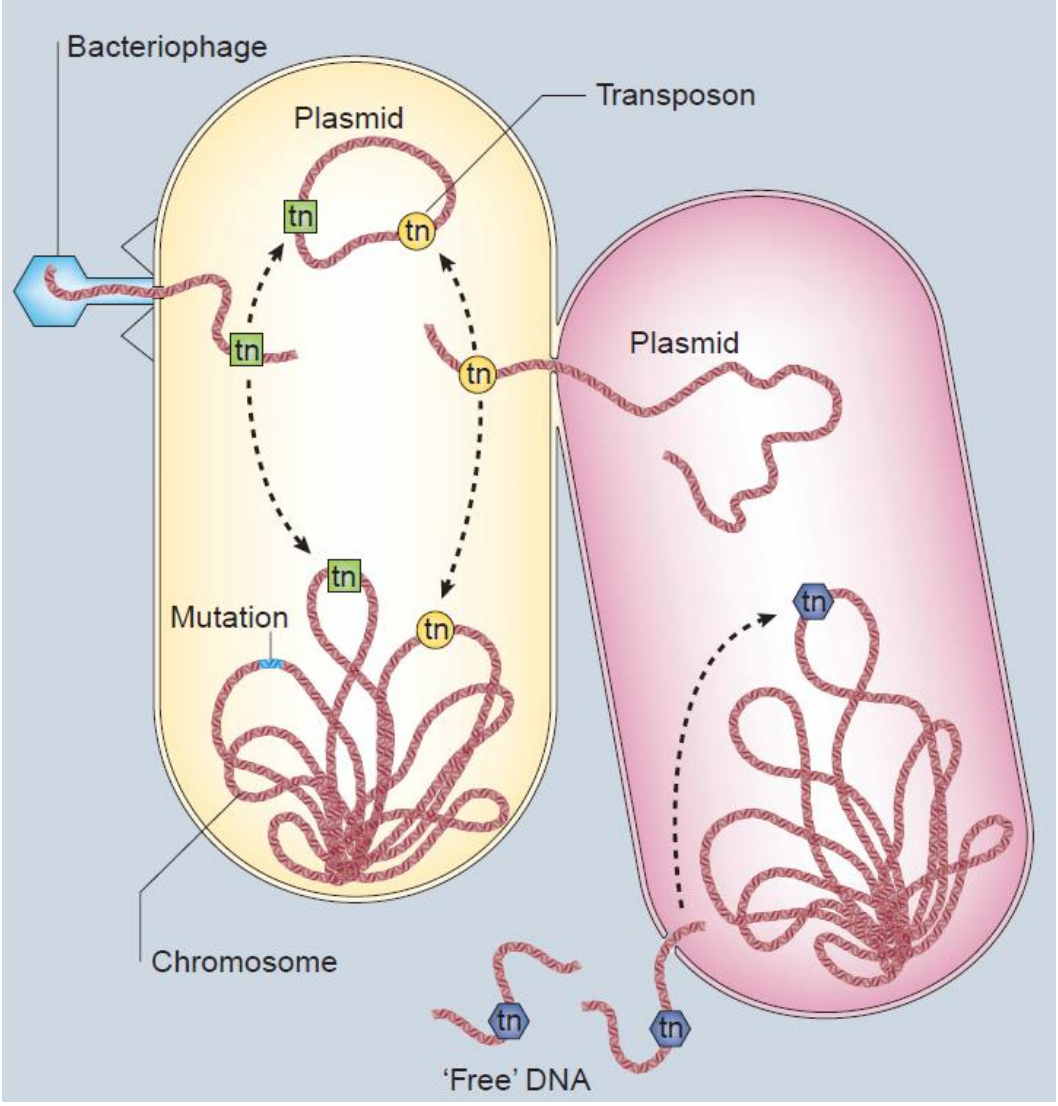

Figure 1 - Mechanisms of bacterial gene transfer (Source: Levy and Bonnie 2004).

It is expected that appearance of resistance against new antimicrobial compounds continues to be a major problem. Additionally, in clinical environments, there has been an increasing number of novel resistance genes mainly because of the uncontrolled use of antibiotics in the past years. Nowadays, there are several strains that are resistant to all the available antibiotics, designated multidrug-resistant bacteria (MDR) or "superbugs". For example, methicillin-resistant (MRSA) *Staphylococcus aureus*, MDR *Acinetobacter baumannii* and numerous strains of extended-spectrum β-

lactamase (ESBL)-producing such as *Escherichia coli, Salmonella* spp., *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. For instance, MRSA *S. aureus* is the main cause of nosocomial and society-acquired infections it causes bloodstream infections, pneumonia and surgical-site infections, and is resistant to methicillin and other penicillin class antibiotics (Alnahdi 2014).

In two studies conducted in Lechuguilla cave (Bhullar 2011), researchers compared surperficial and subterranean isolates of *Paenibacillus* spp. and found that non-pathogenic bacteria contain several different genes orthologous and even similar to those that are present in pathogenic bacteria. More specific, a single studied bacterial strain was resistant to 26 of 40 tested antibiotics, containing 12 orthologous of known resistance families and 5 new mechanisms of resistance (Pawlowski et al. 2016).

3- New antimicrobial compounds prospection

In search for new therapeutic drugs, there is a huge need for novel and more effective antimicrobial compounds to combat the persistent appearance of multi-drug resistant pathogens (Monciardini et al. 2014). Natural products constitute the major structural and chemical diversity that cannot be compared to any synthetic compound's library of small molecules. They are a continuing inspiration to novel discoveries in scientific areas such as chemistry, biology and medicine. Also, natural products are evolutionarily optimized as drug-like molecules and remain the best sources of therapeutic drugs (Newman and Cragg 2012).

In order to increase the odds on prospection of antimicrobial compounds, some authors suggest that looking in less studied and neglected habitats may be a good strategy to find novel compounds (Monciardini et al. 2014). Also, many of the available antibiotics were isolated from microorganisms namely, bacteria and fungus (Etebu and Arikekpar 2016). Since only a small fraction of microorganisms has been screened for the production of bioactive molecules, there is, in principle, a rich, unexplored source for specialized microorganisms and consequently their metabolites (Monciardini et al. 2014). This has a special interest in exploring new microbial taxa, different niches and poorly explored habitats such as

subterranean environments. As an example, a larger part of the discovered antimicrobial compounds has been isolated from terrestrial environments, but recently almost 23% of the new bioactive molecules were isolated from unexplored marine niches such as sediments (Cragg and Newman 2014), sponges (Anand et al. 2006) or the deep-sea (Anand et al. 2006).

Additionally, the subterranean environment could be considered an extreme habitat due the oligotrophic conditions that it features. The lack of nutrients makes the life in caves intolerable for most of the microorganisms (Barton 2006). Microorganisms living in extreme conditions have to be considered a good source to find new bioactive compounds (Wilson and Brimble 2009) This make the subterranean environment the perfect location to increase the odds on prospection for new antimicrobial compounds.

4- Caves as a suitable environment for prospecting microorganisms with novel antibiotic compounds

4.1- Caves

Caves are defined as natural spaces below the Earth's surface that are accessible to human entrance (Gillieson 1996). Speleogenesis is any natural process that hollow out of rock resulting in the formation of natural caves. These processes include dissolution, volcanic activity, mechanical weathering or the melting of glacial ice (Engel 2010).

The classification of natural caves is mainly based on the type of host solid rock where they were developed and on the method of formation. Concerning the geological constitution, the most common caves are those formed in limestone, calcareous and basaltic rocks. There are also other type of caves such as gypsum, granite, talus, quartzite, ice and sandstone, but are usually less extensive and less frequent (Ford and Williams 2013).

Regarding the formation process there are three main primary mechanisms: dissolution by carbonic acid, the dissolution driven by sulfuric acid and the cooling of lava from volcano eruption. The typical limestone caves are formed when water passes through the soil, taking in carbon dioxide and forming a dilute solution of carbonic acid. As this water infiltrates the ground, the direct contact with

limestone makes the water dissolve calcium carbonate. When the water reaches the cave, carbon dioxide is released, allowing the formation of stalactites and stalagmites (Engel 2010). Altamira cave in New Spain and Krubera cave, the deepest cave in the world, in Georgia, are classical examples of limestone caves. Cave formation driven by sulfuric acid occurs when hydrogen sulphide progresses along fissures and until reaches the oxygenated zone. At the oxygenated zone, some forms of sulfuric acid can dissolve limestone. Carlsbadd caves and Lechuguilla caves are examples of sulfuric acid cave type. Lastly, lava cave tubes occur when during a volcano eruption the superficial molten lava cools down faster than the interior. When the eruption stops, the flowing lava solidifies leaving the interior conduit empty. One of such examples is the Kazumura in Hawai being the longest lava tube in the world and also in Azores the lava tube named Torres cave (Northup and Lavoie 2001).

4.1.1- Cave zonation

Caves are zonal environments with zonation in caves being defined based on several physical parameters such as the amount of light, moisture, air flow, gas concentration and evaporitic power of the air (Ghosh, Kuisiene, and Cheeptham 2016). Based on these parameters, we can define five different zones: entrance, twilight, transition, deep and stagnant air zones.

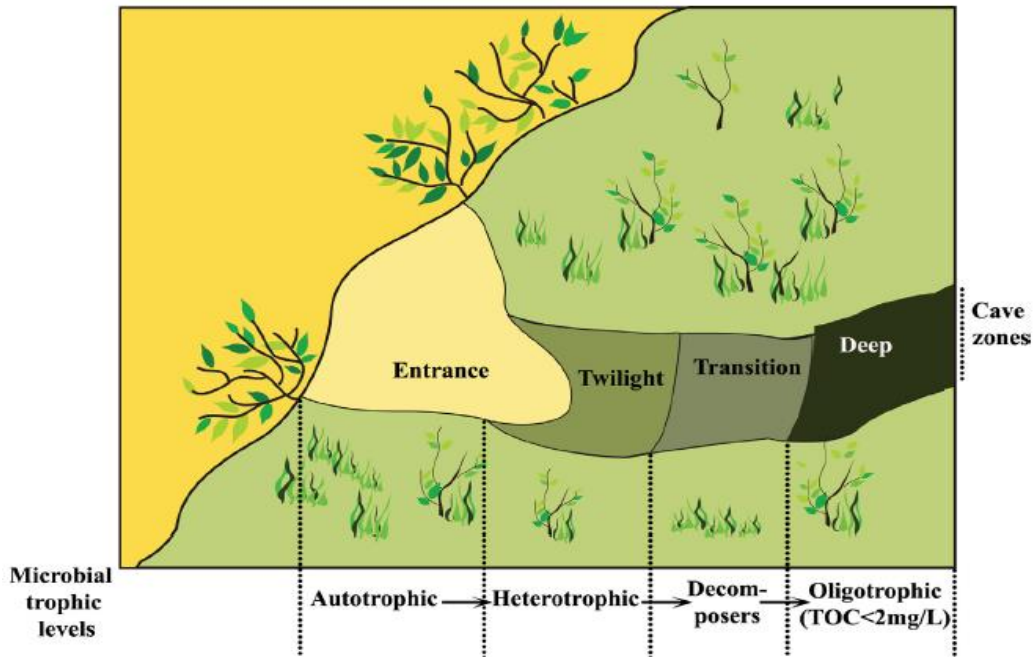

Figure 2 - Schematic representation of cave zones. (Source: Ghosh, Kuisiene, & Cheeptham, 2016).

The entrance and twilight zones are the most influenced by surface. These areas are the limit for growth of vascular green plants once sun light can reach these zones. Transition zone is characterized by total darkness and some variation regarding the other abiotic parameters (fluctuations in air flow, moisture and potential evaporation rates). At the deep cave zone, the physical parameters remain very stable: air is saturated with water vapor and the substrate is constantly moist. The stagnant air zone is not always present and is characterized by restriction in gas exchange causing the stagnation of cave atmosphere (Lurdes and Dapkevicius 2013).

4.1.2- Karstic caves

Karstic landscape is characterized by closed depressions, deep groundwater circulation, dolomitic limestone, evaporitic terrains and caves (Ford and Williams 2013). Karstic landscapes are formed by dissolution of the rock mainly limestone. But dissolution can occur in other type of rock particularly carbonates such as dolomite, in evaporites such as gypsum, in silicates, some basalts and granites.

Consequently, the superficial area of karst terrain (epikarst) is dry and development of dissolution morphologies is very characteristic. These features promote rapid infiltration of water and cause the formation of polja, swallow holes and caves.

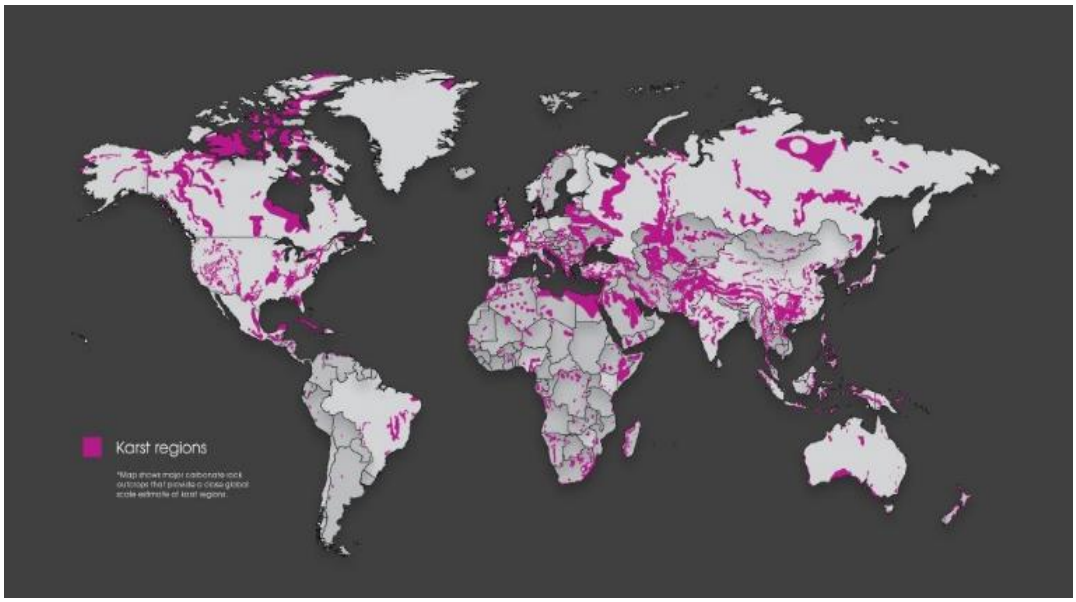

Figure 3 - Karst regions of the world (Source: Ganter, 2018).

Around 15% of world surface is karst and in figure 3 we can see the distribution of karstic terrains worldwide. We can see significant areas in Asia, Europe and in the American continent. Some of these areas and formations are very touristic but a large part remans unexplored (Hollingsworth et al. 2007).

In Portugal the karstic terrain is concentrated in the mainland (figure 4), being the most relevant karst areas the Estremenho (Serra de Aire e Candeeiros), Arrábida, Sicó-Condeixa-Alvaiázere, Montejunto and Algarve. These areas are Jurassic limestone and dolomites and caves are found from the costal territory (Arrábida) to higher points like Serra de Montejunto (Reboleira et al. 2011)

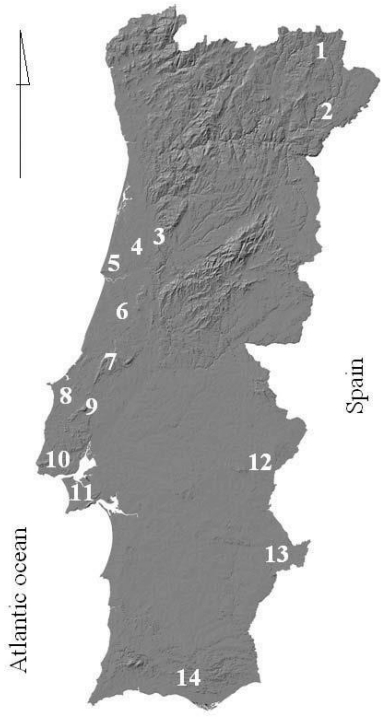

Figure 4 - Karstic regions of Portugal. (1- Dine; 2- Vimioso; 3- Cantanhede; 4- Mealhada; 5- Boa Viagem;6- Sicó-Condeixa e Alvaiázere; 7- Estremenho; 8- Cesaredas; 9- Montejunto; 10- Península de Lisboa; 11- Arrábida; 12- Estremoz-Cano; 13- Adiça-Ficalho; 14- Algarve.) (Source: Reboleira et al. 2011).

4.2- Cave bacteria

Study of cave bacteria is relatively recent and consequently limited. The ecological role of these organisms in subterranean environments isn't entirely determined yet, but some groups have already been related to some functions. Regarding the ecological role of cave bacteria two functions were predicted by

scientists: bacteria as primary producers and bacteria altering cave shape. For example, some sulphur-oxidizing bacteria were associated with gypsum and carbonate formation (Cacchio et al. 2004). Also, chemolithotrophic bacteria are able to gain energy by using sulphur molecules as electron donors, acting like primary producers. Another example is ammonium and nitrite oxidizing bacteria that were associated with saltpeter formation (Kumaresan et al. 2014). These bacteria can convert ammonia into nitrate allowing deposition of saltpeter and modelling the cave shape (Barton 2006).

As for diversity, the phylum Proteobacteria is one of the most representative and members of all the five sub-groups were found in cave environments. Also, members of Actinobacteria, Firmicutes, Bacteroidetes, Flavobacteria and Nitrospira were detected in all three types of caves: limestone, granitic and lava (Tomczyk-Żak and Zielenkiewicz 2016). To study diversity of cave bacteria there are two main approaches: cultivation methods and molecular phylogenetic analysis (Barton 2006). Cultivation approaches are very limited, however having bacterial strains in laboratory allows investigators to perform several tests, adjust cultivation conditions and extract bioactive molecules produced by these microorganisms (Gálvez et al. 1993; Lamprinou et al. 2015). Nonetheless, scientists believe that 99% of the existent bacteria are non-cultivable (Cacchio et al. 2004). Molecular approaches allow an overall view of the diversity and also allow the prediction of some ecological roles of distinct bacterial groups in which cultivation methods are ineffective (Lurdes and Dapkevicius 2013).

4.2.1- Cultivable diversity of cave bacteria

Cultivation methods can be very limited for having a real picture of bacterial diversity in caves, mainly because there are no standard methodologies for cultivation of this type of cells and the wide variety of studies in this area. The type of samples is the primary variable in studies on cave bacteria. Samples could range from soil and sediment (Nakaew, Pathom-aree, and Lumyong 2009), rock walls (Tomova et al. 2013), moonmilk (Portillo and Gonzalez 2011), biofilms (Borsodi et al. 2012), coloured spots on walls (Porca et al. 2012) to Palaeolithic paintings (Bastian et al. 2010). The culture media employed are very variable too, as well as the incubation period, temperature and pH. Studies also have different aims and some only intent to isolate a fraction of bacterial diversity. Table 1

summarizes some studies carried out in caves and specify the genera found in those studies.

Table 1- Studies on cultivable bacteria isolated from caves.

Type of sample	Identified genera	Reference
Moonmilk speleothem, water of underground lake	*Streptomyces; Nocardia*	(Axenov-Gribanov et al. 2016)
Soil samples	*Actinomadura; Actinoplanes; Gordonia; Microbispora; Micromonospora; Nocardia; Nonomureae; Saccharopolyspora;*	(Niyomvong et al. 2012)
Several types of samples	*Streptomyces; Nocardia; Rhodococcus; Nocardioides*	(Groth et al. 1999)
Sediments	*Bacillus; Pseudomonas; Brevibacillus; Enterococcus; Paenibacillus; Microbacterium; Phenylobacterium; Caulobacter; Sphingomonas; Exiguobacterium; Massilia; Psychrobacter; Carnobacter; Staphylococcus*	(Yasir 2018)
Soil samples	*Streptomyces*	(Belyagoubi et al. 2018)
Guano deposits, log and twig deposits, leaf litter deposits	*Brevundimonas; Bacullis*	(Karkun, Patle, and Verna 2014)
Biofilm samples	*Geothermobacterium; Levilinea; Nitrospira; Ignavibacterium; Desulfovibrio; Anaeromyxobacter; Thermolithobacter; Acidothermus; Lentzea; Cytophaga; Methylacidiphilum*	(Borsodi et al. 2012)
Soil and clay from cave walls, sediment, speleothems, drinkable water and coloured spots in cave walls	*Micrococcus¸Bacillus*	(Klusaite et al. 2016)
Rock walls	*Enterobacter; Pseudomonas; Serratia; Bacillus; Micrococcus; Sphingobacterium; Acinetobacter; Obesumbacterium; Arthrobacter Stenotrophomonas; Comamonas*	(Tomova, Lazarkevich, Tomova, Kambourova, & Vasileva-Tonkova, 2013)
Yellow, grey, pink and white cave silver	*Paenibacillus; Pseudomonas; Lysobacter; Sphingomonas; Bosea; Agrobacterium; Micrococcus; Bacillus; Oerskovia;*	(Velikonja, Tkavc, and Pašić 2014)

	Arthrobacter; Streptomyces; Aerococcus	
Soil samples	*Streptomyces; Micromonospora; Spirillospora; Saccharothrix; Nonomuraea; Actinocorallia; Pseudonocardia; Catellatospora; Microbispora*	(Nakaew, Pathom-aree, and Lumyong 2009)
Moonmilk samples	*Agromyces; Amycolatopsis; Kocuria; Micrococcus; Micromonospora; Nocardia; Streptomyces; Rhodococcus*	(Nakaew, Pathom-aree, and Lumyong 2009)

Prospecting new antimicrobial compounds from cave bacterial isolates

Studies on antimicrobial activity of cave isolates has increased in the past decade (Yasir 2018; Patel et al. 2014; Bredholdt et al. 2007; Yücel and Yamaç 2010). Many investigations were conducted in cave environments due to the abiotic pressure that is a feature in caves. Together with the relatively low and constant temperature, absence of light makes impossible the growth of vascular green plants and consequently the amount of organic matter is very low. At these circumstances, microorganisms need to be adapted to limitations on available nutrients closed to starvation (Barton and Northup 2007). At the same time, bacteria need to control the growth of other bacterial groups producing different types of antimicrobial molecules that could be used as antibiotics.

Methods for evaluating antimicrobial activity aren't unanimous and each investigation employs different methodologies. For example, a study conducted in two caves of Pakistan used confrontation bioassay with paper filter discs to evaluate the antimicrobial potential of their isolates (Yasir 2018). In contrast, another study that evaluated the antimicrobial capacity of *Streptomyces* spp. isolated from Grotte des Collemboles (Springtails'Cave) in Belgium choose cross-streak method (Maciejewska et al. 2016).

Antimicrobial activity of cave isolates has been studied in several caves around the world. For instance, from a collection of 47 actinomycetes isolated from Chaase cave in Algeria, all showed antimicrobial activity against at least one test agent and 61,7% were active against *S. aureus* (Belyagoubi et al. 2018). In other study performed in two caves in the Hindu Kush mountain, 86 isolates were

tested against *Salmonella thypimurius* and *S. aureus* and the results revealed that 15 and 30 were respectively active against these test agents. Also, six isolates were active against both pathogens (Yasir 2018). In an investigation conducted in Krubera-Voronja Cave, it was possible to isolate two different compounds extracted from two *Bacillus* strains, suggesting that bioactivity assays should be performed using bacterial isolates from Firmicutes phylum contrasting with of focus given by many authors for Actinobacteria (Klusaite et al. 2016).

5- Goals and structure of the dissertation

Considering the necessity for new antibiotics and the increased potential for obtaining these from microorganisms growing in relatively unexplored environments, such as caves, the main objectives of this dissertation were to:

i) Isolate cultivable bacteria from Algarve caves and identify them based on 16S rDNA gene sequence;

ii) Screen bacterial isolates for antimicrobial activity against six test agents: *Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 27853*, Staphylococcus aureus* ATCC 25923*, Enterococcus faecalis* ATCC 29212*, Aeromonas salmonicida* ATCC 33658 *and Bacillus cereus* ATCC 14579.

The present dissertation is composed of three chapters. The first is of introductory nature, providing information over the various aspects relevant for understanding the global perspective of this thesis. The second chapter comprehends the practical approach in order to attain the objectives of this dissertation, and the final chapter (III) consists of the final remarks, where all of the results obtained are further discussed and future perspectives are addressed.

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II - Diversity and antimicrobial potential of bacteria isolated from Algarve caves

1- Introduction

In the past decades, there has been an increasing demand for new antibiotics due to the ability of microorganisms to develop resistances against existing antibiotics. The World Health Organization (WHO) stated global health emergency to the current multiplying resistance strains and the lack of new antibiotic to fight them (World Health Organization 2014). Along with this, scientific community dedicate their attention to explore neglected and extreme environments to enlarge natural product drug discovery (Onaga 2001). For example, Vollú et al. (2014) found that 13,7% of the isolates of spore-forming bacteria isolated from Antarctic soil samples were able to inhibit the growth of MRSA. Also, another study conducted in the Thar Desert revealed a remarkable antimicrobial activity of an yellow pigment extracted from *Streptomyces hygroscopicus* subsp. *ossamyceticus* (Selvameenal, Radhakrishnan, and Balagurunathan 2009).

Caves are considered extreme environments for the occurrence of life forms. The reunion of severe abiotic conditions such as total darkness, thermal stability and high humidity (98-100%) results in an absence of primary producers and consequently starved conditions (Barton 2006). Also, cave environments have very low organic carbon input since energy enters the cave in a very limited way, only via entrances, sinkholes, dripping waters or underground hydrology (Diana E Northup and Lavoie 2001). These circumstances make caves only capable of sustaining highly specialized microorganisms and their metabolic pathways are augmented over the bioactive molecules production inhibiting the growth of other microorganisms (Lurdes and Dapkevicius 2013). For instance, from a cave soil sampled collected in the mountain of Miroc in Serbia, Stankovic and her colleagues isolated a red-pigment producing bacterium. The tests conducted on the deep red pigment revealed antimicrobial activity against *Bacillus* and *Micrococcus* species and *Candida albicans*, but also antioxidative and UV-protective properties (Stankovic et al. 2012).

One of the most common types of caves are karstic caves. They are extensively distributed worldwide, many of them are yet to be discovered or have hardly been accessed, although other caves with pre-historic paintings have received substantial attention (Groth et al. 1999; Tomova et al. 2013). Despite

their historically importance, our knowledge on the microbiology diversity of caves is still scarce (Tomova et al. 2013). Namely in the Portuguese territory, despite the existence of large karstic massifs encompassing the majority of the existing caves systems, there are no studies concerning microorganisms.

Studies on cultivable diversity of cave bacteria encompasses members of *Proteobacteria*, *Actinobacteria* and *Firmicutes* groups (Tomczyk-Żak and Zielenkiewicz 2016). Current molecular techniques revealed a great microbiological diversity in cave environments, surprisingly high compared to what was expected given the amount and complexity of nutrients available in subterranean environments (Wu et al. 2015). A metagenomic study performed in Villa Luz cave revealed a surprisingly diversity encompassing seven phyla namely, *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Ignavibacteria and Proteobacteria* (D'Auria et al. 2018). Besides the diversity studies, scientists have also dedicated their time to develop new techniques to discover unknown secondary metabolites, especially those with antimicrobial properties (Donadio et al. 2002).

There are some studies that have already evaluated the antimicrobial activity of bacterial isolates inhabiting subterranean environments, revealing a great potential. Such has reinforced the ideia that there is a great potential on prospecting of novel bioactive compounds in these habitats (Niyomvong et al. 2012; Rule & Cheeptham 2013; Nareeluk Nakaew et al. 2009).

Considering the constant need for new antibiotics, the potential of cave habitats for prospection of novel compounds, and the lack of studies in the Portuguese territory, the main aim of this study was to isolate bacteria from Algarve caves, identify them, and verify if any are capable of presenting antimicrobial activity against six test agents: *Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa* ATCC 27853*, Staphylococcus aureus* ATCC 25923*, Enterococcus faecalis* ATCC 29212*, Aeromonas salmonicida* ATCC 33658 and *Bacillus cereus* ATCC 14579*.*

2- Materials and Methods

2.1- Sampling site

Algarve is the southest region of mainland Portugal. The region has one of the most important karstic massifs with more than fifty natural caves known.

This study was conducted in three caves of the Algarve region: Ibne Ammar, Vale do Telheiro and Senhora (see figure 1). The Ibne Ammar cave is located in Lagoa city, on the left side of the Arede river. The cave has many entrances and some galleries are flooded due to the tide effect. As for Vale do Telheiro cave it is one of the largest caves in the region and it's located in Loulé city. Senhora cave is part of a geological monument named Cerro da Cabeça, located in Olhão.

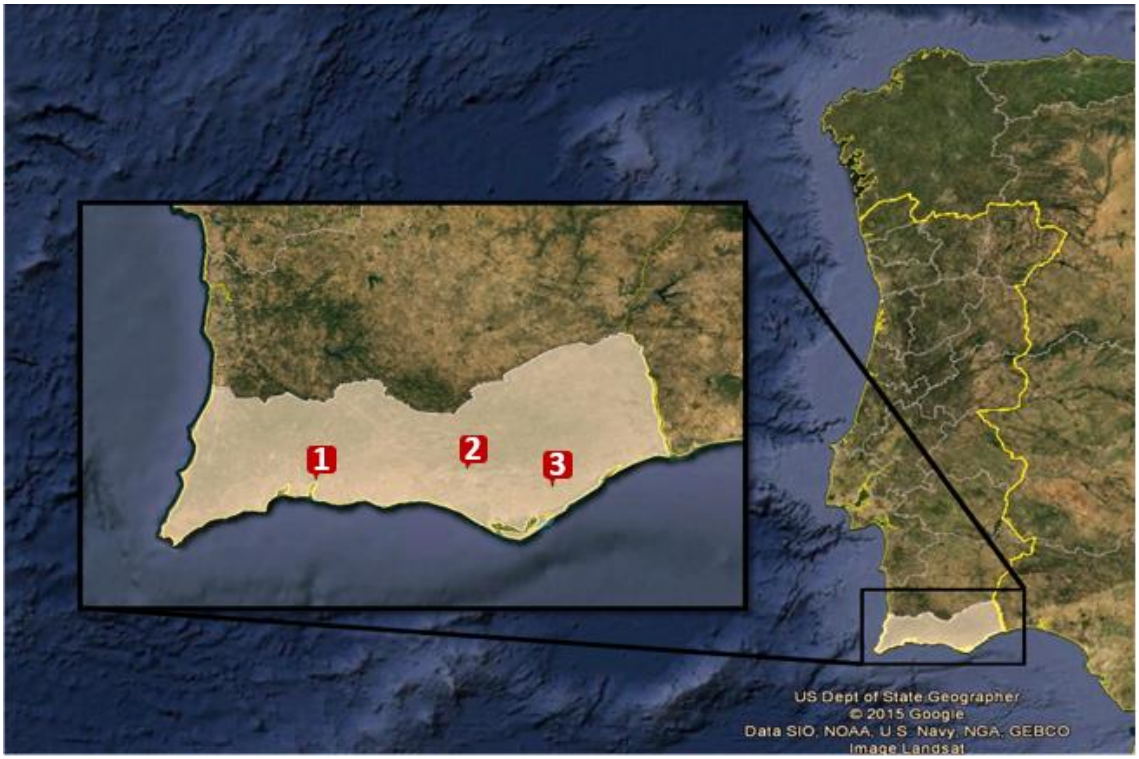

Figure 1 - Location of Algarve caves (1- Ibne Ammar cave; 2- Vale do Telheiro cave; 3- Senhora cave).

The access of all the sampled caves was very difficult and requires speleological support material. All the galleries selected for sampling had no evidences of human presence in order to assure that bacterial samples were really from subterranean environments. Also, no major sources of organic matter were observed in all the three caves. In the entrance zone we are able to see some superficial roots and very few bats (one or two), but at deeper galleries, where samples were taken, we could only see insects. The entrance areas

present superficial roots and bats, but in the deeper galleries, where samples were taken, only invertebrates were observed.

2.2- Sampling, isolation and culture conditions

Samples were collected by gently passing on cave walls with sterile cotton swabs. Immediately after, the samples were inoculated on Petri dishes with TSA and PCA media and falcon tubes with TSB, and kept refrigerated until arrival to the laboratory. At the laboratory, samples on TSB tubes were transferred to Petri plates with TSA. All samples were kept at room temperature with no light. When bacterial growth was evident, isolation of all morphological different colonies was carried out for a new petri plate with TSA media. Pure cultures were maintained in microtubes (in triplicates) in a solution of TSB with 20% of glycerol, at –20ºC.

2.3- Amplification of 16S rDNA gene by PCR and sequencing

To allow the sequencing analysis, twenty-four hours broth cultures of all isolates were prepared for DNA extraction using GF1 bacterial DNA extraction kit (Vivantis Technologies). Genomic DNA was used as template DNA for 16S gene PCR amplification. Reactions of 25 µL containing: The PCR reactions were performed in 25 µL reactions containing 0.2 µM each primer (27F and 1492R) 1x PCR buffer, 0.2 mM each dNTP, 2 mM $MgCl_2$,1U Taq polymerase and 2 µl of cell lysate as template DNA. The reaction mixture was incubated in a Thermal Cycler (Bio-Rad) with an initial denaturation step at 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, then a final extension step at 72 °C for 5 min.

The PCR products were then purified and sequenced through Sanger sequencing at STAB Vida laboratories (Caparica, Portugal) using universal bacterial primers 27F and 1492R. Upon assembly in SeqTrace, all sequences were compared to database available from GenBank to identify the most similar strain.

2.4- Screening test for antimicrobial activity

Only isolates that grew in a 24h of period were selected for the antimicrobial test screening. Six test agents were used (three Gram positive and three Gram negative bacteria): *Escherichia coli* ATCC 25922*, Pseudomonas aeruginosa*

ATCC 27853*, Staphylococcus aureus* ATCC 25923*, Enterococcus faecalis* ATCC 29212*, Aeromonas salmonicida* ATCC 33658 *and Bacillus cereus* ATCC 14579*.* The test agents grew on TSA media, at 37ºC and at a density of 0.5 McFarland were inoculated with a cotton swab on a large Petri plate with Mueller Hinton agar. After five minutes, 5 µL of the cave bacterial isolates suspensions (0.5 McFarland) were inoculated in the same plate in equidistant points. After 24h to 48h of incubation, the diameter of the inhibition zone was measured. Tests were carried in triplicate at room temperature.

3- Results

3.1- Cultivable bacterial diversity

A total of 110 bacterial isolates were isolated from the three sampled caves of Algarve region. Fifty-two were recovered from Vale do Telheiro cave, forty-two from Senhora cave and sixteen from Ibne Ammar cave. All the bacterial isolates are listed in table 1 as well as the corresponding cave of origin, the type of media used in the first inoculation, the percentage of query cover, the percentage of identity, the sequence length in base pairs and the closest relative strain deposited in GenBank.

Table 1 - List of bacterial isolates of Algarve caves.

Isolate code	Cave	Type of cultivation media	Query cover (%)	Identity (%)	Sequence length (bp)	Closest relative strain (GenBank)
704	Vale do Telheiro	TSB	100	99	1422	*Viridibacillus arvi* LMG 22165
705	Ibne Ammar	TSB	100	100	1422	*Bacillus mobilis* MCCC 1A05942
706	Senhora	TSB	100	99	1424	*Bacillus mobilis* MCCC 1A05942
707	Vale do Telheiro	TSB	100	100	1058	*Lysinibacillus parviboronicapiens* NBRC 103144
708	Vale do Telheiro	TSA	100	99	1406	*Bacillus mycoides* NBRC 101228
709	Vale do Telheiro	TSA	100	97	1150	*Lysinibacillus parviboronicapiens* NBRC 103144
710	Senhora	TSA	100	99	1343	*Ochrobactrum pecoris* 08RB2639
712	Senhora	TSA	100	99	1384	*Serratia ficaria* JCM1241
713	Vale do Telheiro	TSA	100	99	1419	*Brevibacterium frigoritolerans* DSM 8801
715	Vale do Telheiro	TSA	100	99	1415	*Bacillus mycoides* NBRC 101228
716	Senhora	TSA	100	98	1394	*Viridibacillus arvi* LMG 22165
718	Senhora	PCA	100	99	1373	*Bacillus proteolyticus* MCCC 1A00365
720	Vale do Telheiro	PCA	100	99	1405	*Paenibacillus lautus* AB236d

721	Vale do Telheiro	PCA	100	99	1416	*Paenibacillus taichungensis* BCRC 17757
724	Vale do Telheiro	PCA	100	92	1424	*Lysinibacillus parviboronicapiens* NBRC 103144
725	Vale do Telheiro	TSA	100	99	1421	*Bacillus mycoides* NBRC 101228
728	Vale do Telheiro	TSA	100	99	1422	*Bacillus mycoides* NBRC 101228
729	Vale do Telheiro	TSB	99	99	1345	*Lysinibacillus parviboronicapiens* NBRC 103144
731	Senhora	TSB	100	100	1410	*Bacillus mycoides* NBRC 101228
732	Senhora	TSB	100	100	1383	*Bacillus proteolyticus* MCCC 1A00365
733	Vale do Telheiro	PCA	100	99	1413	*Serratia quinivorans* 4364
734	Vale do Telheiro	PCA	100	99	1286	*Serratia quinivorans* 4364
740	Ibne Ammar	PCA	100	100	1384	*Nocardia coeliaca* DSM 44595
743	Vale do Telheiro	PCA	100	100	1407	*Viridibacillus arvi* FJAT-45874
744	Vale do Telheiro	PCA	100	99	1421	*Brevibacterium frigoritolerans* DSM 8801
748	Vale do Telheiro	PCA	100	100	1373	*Bacillus proteolyticus* MCCC 1A00365
750	Vale do Telheiro	PCA	100	100	1424	*Bacillus mycoides* NBRC 101228
752	Vale do Telheiro	PCA	100	100	1389	*Bacillus mycoides* NBRC 101228
753	Vale do Telheiro	TSA	100	99	1391	*Pseudomonas brenneri* CFML 97-391
754	Vale do Telheiro	TSA	100	99	1402	*Pseudomonas chlororaphis* subsp. *aurantiaca* NCIB 10068
756	Vale do Telheiro	TSA	100	99	1412	*Bacillus mycoides* NBRC 101228
758	Senhora	PCA	100	99	1385	*Paeniglutamicibacter kerguelensis* KGN15
760	Senhora	TSA	100	100	1383	*Brevibacterium frigoritolerans* DSM 8801

761	Senhora	TSA	100	99	1416	*Bacillus mobilis* MCCC 1A05942
763	Vale do Telheiro	PCA	100	99	1419	*Bacillus wiedmannii* FSL W8-0169
764	Vale do Telheiro	PCA	100	99	1357	*Rhodococcus jialingiae* djl-6-2
765	Vale do Telheiro	PCA	100	99	1328	*Achromobacter marplatensis* B2
766	Senhora	PCA	100	100	1413	Bacillus wiedmannii FSL W8-0169
767	Senhora	PCA	100	100	1434	*Bacillus mycoides* NBRC 101228 16S
768	Senhora	PCA	100	100	1386	*Bacillus wiedmannii* FSL W8-0169
769	Senhora	PCA	100	99	1443	*Bacillus wiedmannii* FSL W8-0169
770	Senhora	PCA	100	99	1439	*Bacillus wiedmannii* FSL W8-0169
771	Ibne Ammar	TSA	100	99	1418	*Bacillus mobilis* MCCC 1A05942
772	Ibne Ammar	TSA	100	99	1415	*Lactococcus taiwanensis* 0905C15
773	Ibne Ammar	TSA	100	99	1403	*Bacillus toyonensis* BCT-7112
774	Senhora	TSB	100	100	1427	*Psychrobacillus lasiicapitis* NEAU-3TGS17
775	Senhora	TSB	100	100	1403	*Brevibacterium frigoritolerans* DSM 8801
776	Senhora	TSB	100	98	1429	*Sporosarcina psychrophila* NBRC 15381
777	Senhora	TSA	100	99	1445	*Pseudomonas chlororaphis* subsp. *aurantiaca* NCIB 1006
780	Senhora	TSB	100	99	1422	*Psychrobacillus lasiicapitis* NEAU-3TGS17
782	Vale do Telheiro	TSA	100	99	1418	*Bacillus mycoides* NBRC 101228
786	Senhora	TSA	100	99	1421	*Psychrobacillus lasiicapitis* NEAU-3TGS17

787	Vale do Telheiro	TSA	100	99	1409	*Psychrobacillus lasiicapitis* NEAU-3TGS17
788	Vale do Telheiro	TSA	100	99	1419	*Psychrobacillus psychrodurans* 68E3
793	Ibne Ammar	TSA	100	99	1406	*Bacillus mycoides* NBRC 101228
794	Senhora	TSA	100	99	1400	*Psychrobacillus lasiicapitis* NEAU-3TGS17
796	Vale do Telheiro	TSA	100	99	1404	*Pseudomonas chlororaphis* subsp. *aurantiaca* NCIB 10068
797	Vale do Telheiro	TSA	100	99	1420	*Bacillus mobilis* MCCC 1A05942
798	Vale do Telheiro	TSA	100	99	1405	*Bacillus kochii* WCC 4582
800	Vale do Telheiro	TSA	100	100	1414	*Bacillus mycoides* NBRC 101228
801	Vale do Telheiro	TSA	100	99	1392	*Glutamicibacter arilaitensis* Re117
802	Vale do Telheiro	PCA	100	99	1383	*Serratia grimesii* DSM 30063
803	Vale do Telheiro	PCA	100	99	1412	*Serratia grimesii* DSM 30063
805	Ibne Ammar	TSA	100	99	1416	*Staphylococcus edaphicus* CCM 8730
806	Ibne Ammar	TSA	100	99	1402	*Lysinibacillus contaminans* FSt3A
807	Vale do Telheiro	PCA	100	99	1413	*Serratia grimesii* DSM 30063
808	Vale do Telheiro	PCA	100	99	1429	*Serratia grimesii* DSM 30063
809	Vale do Telheiro	PCA	100	99	1397	Pseudomonas helmanticensis OHA11
810	Vale do Telheiro	TSA	100	100	1416	*Bacillus mycoides* 1-3T
812	Vale do Telheiro	TSB	100	100	1403	*Bacillus safensis* NBRC 100820
813	Ibne Ammar	PCA	100	100	1426	*Bacillus mycoides* NBRC 101228
814	Vale do Telheiro	PCA	100	99	1399	*Serratia liquefaciens* ATCC 27592
816	Ibne Ammar	TSA	100	99	1400	*Advenella kashmirensis* subsp. *methylica* PK1

861	Senhora	PCA	100	99	1387	*Bacillus wiedmannii* FSL W8-0169
862	Senhora	PCA	100	99	1356	*Bacillus mycoides* NBRC 101228
863	Senhora	PCA	100	100	1407	*Bacillus mycoides* NBRC 101228
864	Senhora	PCA	100	99	1402	*Bacillus mobilis* MCCC 1A05942
865	Senhora	PCA	100	99	1230	*Glutamicibacter arilaitensis* Re117
867	Senhora	PCA	100	100	1434	*Bacillus mycoides* NBRC 101228
869	Senhora	TSB	100	100	1404	*Bacillus mycoides* NBRC 101228
871	Ibne Ammar	TSB	100	100	1410	*Viridibacillus arvi* LMG 22165
872	Ibne Ammar	TSA	100	99	1427	*Psychrobacillus* lasiicapitis NEAU-3TGS17
873	Ibne Ammar	TSA	100	99	1386	*Achromobacter kerstersii* LMG 3441
875	Ibne Ammar	TSA	100	99	1389	*Achromobacter kerstersii* LMG 3441
877	Senhora	TSA	100	99	1411	*Stenotrophomonas humi* R-32729
878	Senhora	PCA	100	100	1416	*Bacillus toyonensis* BCT-7112
879	Senhora	PCA	100	100	1388	*Bacillus wiedmannii* FSL W8-0169
880	Senhora	PCA	100	99	1427	*Bacillus wiedmannii* FSL W8-0169
881	Senhora	PCA	100	98	1311	*Lysinibacillus parviboronicapiens* NBRC 103144
882	Senhora	PCA	100	100	1417	*Bacillus mycoides* NBRC 101228
883	Senhora	TSA	100	100	1420	*Bacillus wiedmannii* FSL W8-0169
884	Senhora	TSA	100	100	1424	*Bacillus mycoides* NBRC 101228
886	Vale do Telheiro	TSB	100	99	1311	*Bacillus proteolyticus* MCCC 1A00365
887	Vale do Telheiro	TSB	100	100	1416	*Bacillus mycoides* NBRC 101228

888	Ibne Ammar	PCA	100	100	1421	*Bacillus mycoides* NBRC 101228
890	Vale do Telheiro	TSA	100	100	1423	*Bacillus toyonensis* BCT-7112
891	Vale do Telheiro	TSA	100	98	1424	*Bacillus simplex* LMG 11160
892	Senhora	TSB	100	99	1423	*Bacillus wiedmannii* FSL W8-0169
897	Vale do Telheiro	TSB	100	99	1395	*Viridibacillus arvi* LMG 22165
898	Vale do Telheiro	TSB	100	99	1422	*Viridibacillus arvi* LMG 22165
899	Ibne Ammar	PCA	100	99	1424	*Bacillus toyonensis* BCT-7112
901	Vale do Telheiro	TSA	100	99	1406	*Bacillus mobilis* MCCC 1A05942
902	Vale do Telheiro	TSA	100	100	1410	*Viridibacillus arvi* LMG 22165
904	Vale do Telheiro	TSA	100	99	1386	*Bacillus mobilis* MCCC 1A05942
905	Vale do Telheiro	TSA	100	99	1388	*Bacillus mobilis* MCCC 1A05942
907	Senhora	TSB	100	100	1426	*Bacillus mobilis* MCCC 1A05942
908	Senhora	TSB	100	100	1412	*Bacillus toyonensis* BCT-7112
909	Senhora	TSB	100	99	1318	*Bacillus mobilis* MCCC 1A05942
910	Senhora	TSA	100	100	1418	*Bacillus mobilis* MCCC 1A05942
911	Vale do Telheiro	PCA	100	99	1421	*Bacillus proteolyticus* MCCC1A00365

After 16S gene amplification and sequencing, the bacterial isolates were grouped in three different phyla (figure 2): *Proteobacteria*, *Actinobacteria* and *Firmicutes*, with dominance of the last in all three caves.

Not many differences were found in genus distribution between caves. A total of 19 genera were recovered (figure 3) from all the three caves, namely *Achromobacter, Advenella, Bacillus, Brevibacterium, Glutamicibacter, Lactococcus, Lysinibacillus, Nocardia, Ochrobactrum, Paenibacillus, Paeniglutamicibacter, Pseudomonas, Psychrobacillus, Rhodococcus, Serratia, Sporosarcina, Staphylococcus, Sterotrophomonas* and *Viridibacillus*.

Representatives of genus *Bacillus,* *Lysinibacillus*, *Viridibacillus* and *Psychrobacillus* were common in all caves.

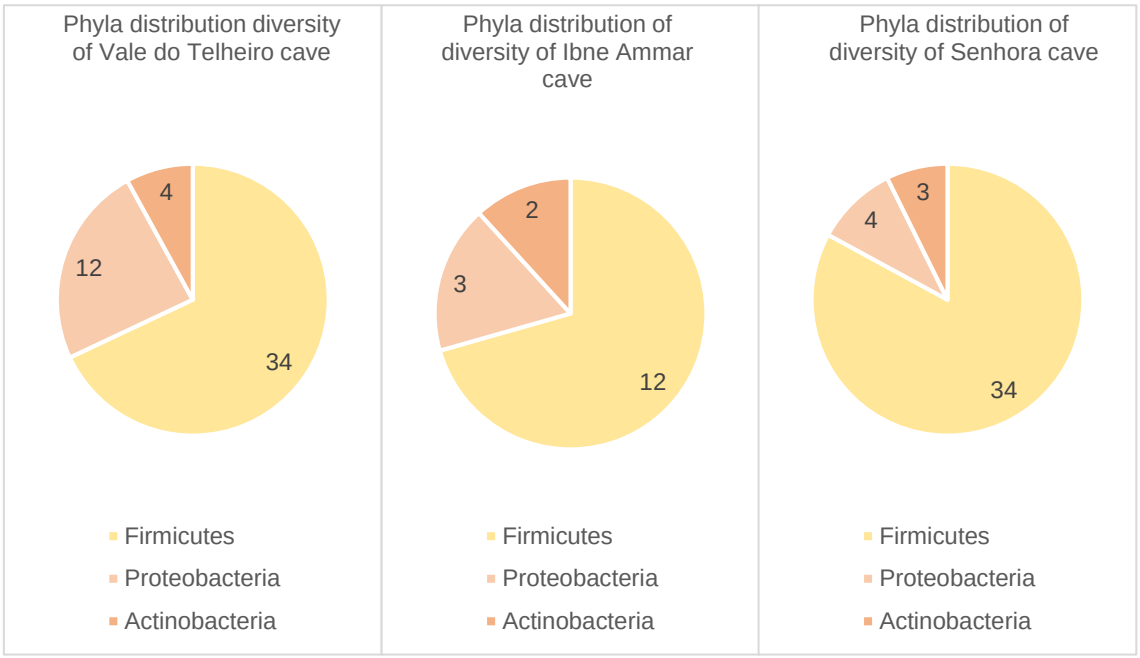

Figure 2 - Phyla distribution of bacteria isolated from Vale do Telheiro, Ibne Ammar and Senhora caves.

From all the sequences analysed it is important to highlight the sequence of the bacterial isolate 724, isolated from Vale do Telheiro sample, since only shared 92% of identity with the closest relative strain *Lysinibacillus parviboronicapiens* strain NBRC 103144. The sequence had 1424 base pairs and 100% in the query cover parameter. This result may suggest a possible new species for *Lysinibacillus* genus.

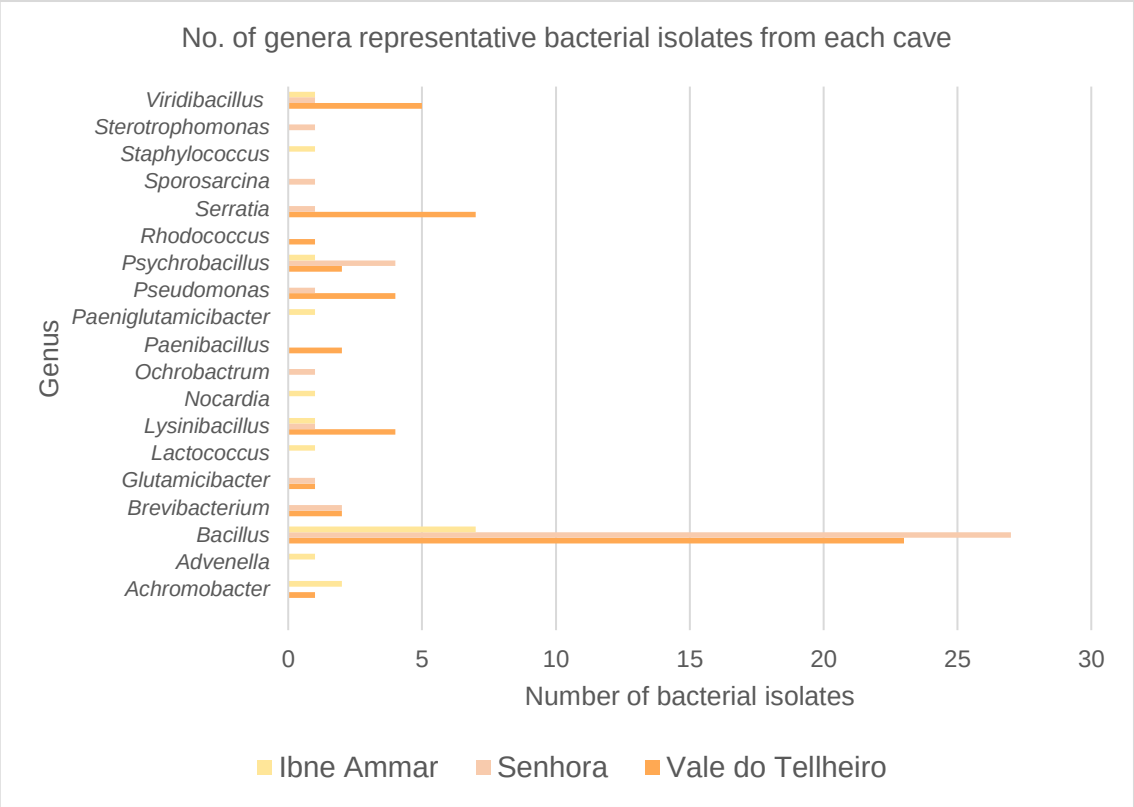

Figure 3 - Genus distribution of bacterial strains according to the cave origin.

3.2- Antimicrobial activity

Approximately 52% of the isolates revealed antimicrobial activity against at least one test agent.

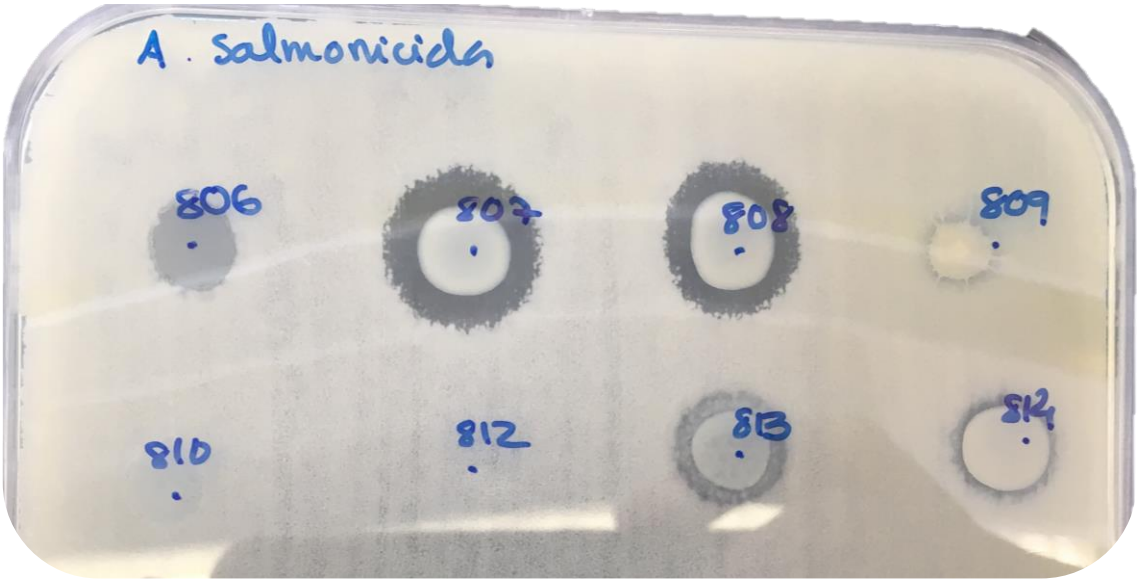

Figure 4 - Example of an antimicrobial test plate with bacterial isolates 807 and 808 showing clear inhibition zones.

The bacterial isolates with activity belonged to the genera *Bacillus, Viridibacillus, Psychrobacillus, Serratia and Pseudomonas.*

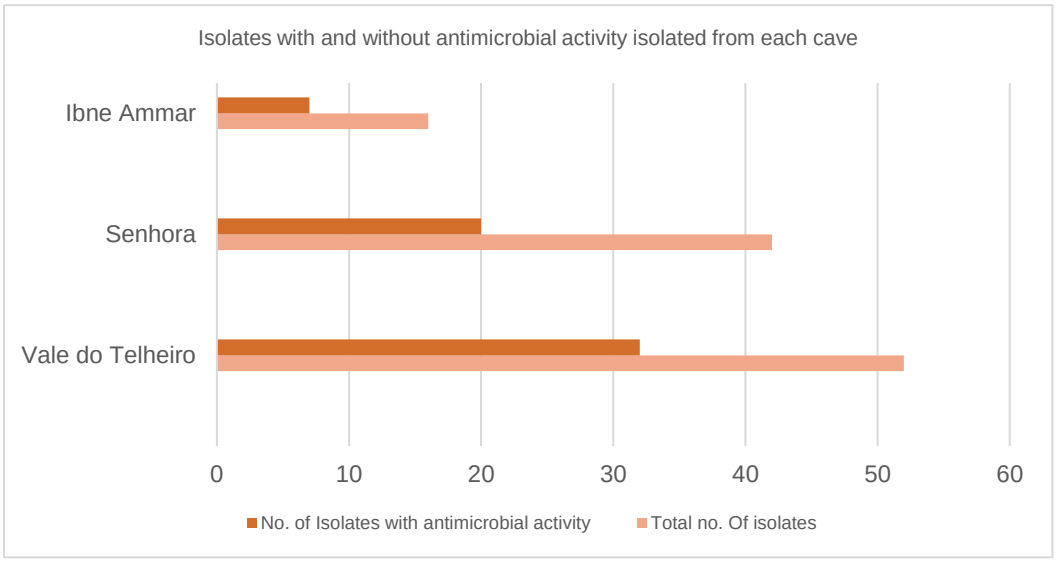

Figure 5 - Antimicrobial activity of bacterial isolates from each cave.

From the total number of strains with antimicrobial activity (figure 6), 81% showed antagonistic activity against *P. aeruginosa*, followed by *B. cereus* (49%), *S. aureus* (47%) and *A. salmonicida* (40%) respectively. Only 10% of the isolates were active against *E. faecalis.* Not a single isolate was active against *E. coli*.

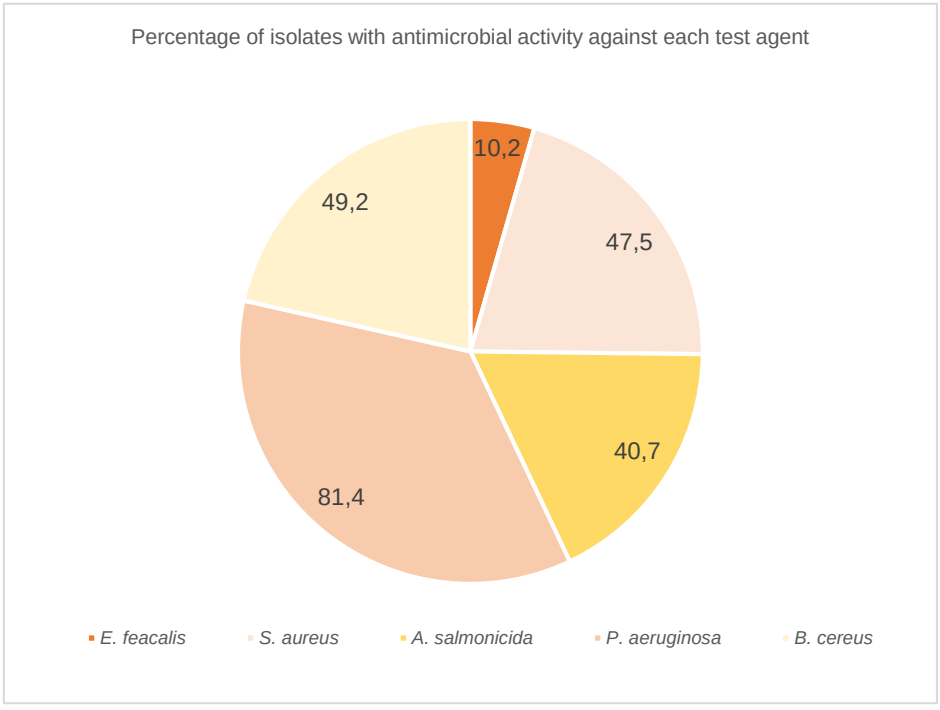

Figure 6 – Percentage of bacterial isolates with antimicrobial activity against each test agent.

A remarkable antimicrobial activity was registered in the ten isolates listed on table 2. These isolates, belonging to genus *Pseudomonas* and *Bacillus,* showed antimicrobial activity against four or five test agents. Isolates 715, 737, 754, 796, 799, 761 and 782 showed antagonistic activity against four test agents.

Isolates 768, 769 and 770 demonstrated a remarkable against five of the six test agents. Isolate 796 registered the major inhibition zones against *S. aureus* (19,54 mm)*, P. aeruginosa* (22,43 mm), *B. cereus* (20,33 mm) and *A. salmonicida* (19,65 mm).

Table 2 - List of strains with remarkable antimicrobial activity.

		Antimicrobial activity agent test				
Isolate code	**Closest relative strain**	***E. feacalis***	***S. aureus***	***P. aeruginosa***	***B. cereus***	***S. salmonicida***
715	*Bacillus mycoides* NBRC 101228	-	++	++	+	+
737	*ND*	++	-	++	+	++
754	*Pseudomonas chlororaphis* subsp. *aurantiaca* NCIB 10068	-	++	++	++	+
768	*Bacillus wiedmannii* FSL W8-0169	+	+	++	+	+
769	*Bacillus wiedmannii* FSL W8-0169	+	+	++	+	+
770	*Bacillus wiedmannii* FSL W8-0169	+	++	++	+	+
796	*Pseudomonas chlororaphis* subsp. *aurantiaca* NCIB 10068	-	++	++	++	++
799	*ND*	-	+	++	++	++
861	*Bacillus wiedmannii* FSL W8-0169	+	++	+	+	-
882	*Bacillus mycoides* NBRC 101228	+	++	++	-	+

ND- Not defined; "-" – no inhibition zone; "+" – Diameter of the inhibition zone < 15mm; "++" – Diameter of the inhibition zone > 15mm.

4- Discussion

Previous studies throughout the world have already demonstrated the potential of caves for harbouring a wide bacterial diversity (Ghosh et al. 2017; Belyagoubi et al. 2018; Borderie et al. 2016) that could present also a new source of molecules with potential bioactivity, namely antimicrobial (Klusaite et al. 2016). However, in Portugal, besides the studies already conducted in the Azorean cave lava tubes (Hathaway et al. 2014). The most significant cave systems existent in the karstic areas, remain unexplored and may represent a valuable asset not only in terms of microbial biodiversity, but also as a potential reservoir for new molecules with bioactivity. Indeed our study, focusing only on cultivable bacteria, showed that karstic Algarve caves, namely Vale do Telheiro, Senhora and Ibne Ammar are inhabited by a great diversity of bacterial groups. Also, using only three types of culture media, TSA, PCA and TSB, we were able to isolate 110 bacterial strains referring to three phyla. Although the number of isolates wasn't really low, the number could be higher if we had employed a poor nutrient media like R2A (Yasir 2018).

The cultured diversity of Algarve caves is dominated by Firmicutes. Our results are in accordance with a previous study performed in Kartchner Caverns (Ikner et al. 2007). In this study Firmicutes were the most represented groups in both touristic and non-touristic impacted areas (Ikner et al. 2007) . Members of the phylum Firmicutes are characterized by a wide range of metabolic capabilities being able to process complex or simple forms of organic compounds. Also, this group is commonly found under extreme conditions like dehydration and nutrient stress (Slepecky and Hemphill 1986). Members of the genus *Bacillus*, the most represented genus, are also able to produce endospores, a resistance strategy to survive adverse conditions (Tomczyk-Żak and Zielenkiewicz 2016). However, the dominance of Firmicutes is not supported by other studies (Aminov 2010; Sarbu, Kane, and Kinkle 1996; Diana E. Northup et al. 2003). For example, in Magura Cave the percentage of Firmicutes was only 6,5% and Proteobacteria was the dominant phyla with 63% (Tomova et al. 2013). In addition, isolate 724 shared only 92% identity with the closest relative strain *Lysinibacillus parviboronicapiens* strain NBRC 103144 (Miwa et al. 2009). The genus was recently proposed (Ahmed et al. 2007) and the type species of the genus is

Lysinibacillus boronitolerans, and its other members are *Lysinibacillus sphaericus* and *Lysinibacillus fusiformis*. This result reinforces the idea that caves may provide novel species increasing the possibility of finding also new molecules.

Proteobacteria were the second largest groups found in Algarve caves. Bacteria belonging to this phylum are dominant in Wind cave (Chelius and Moore 2004), Tito Bustilo cave (Schabereiter-Gurtner et al. 2002) but also on microbial mats of lava caves (D.E. Northup et al. 2011). Like Firmicutes, they can process a wide range of nutrients, being however unable to endure severe nutrient stress (Engel 2010). Presumably, the success of Proteobacteria in subterranean environments could be related to the presence of some insects or organic input via superficial waters infiltration (Tomczyk-Zak and Zielenkiewicz 2015).

Actinobacteria isolated from Algarve caves are represented by genus *Nocardia, Rhodococcus, Brevibacterium, Glutamicibacter and Paeniglutamicibacter*. *Nocardia* and *Rhodococcus* genus are related to degradation of organic matter and decomposition of hazardous chemical compounds (Groth et al. 1999). Bacteria belonging to this phylum often dominate soils microbiota but in subterranean environments they can also be encountered in rock walls and speleothems like stalactites and stalagmites. Some authors believe that members of Actinobacteria are involved in biomineralization processes being, in part, responsible for cave shaping (Cañveras et al. 2001; Jones 2001).

With regard to the antimicrobial capacity of bacteria isolated from Algarve caves, the results showed that more than half of the isolates had antagonistic activity against one of agent tests. Prospection of antimicrobial compounds on less studied and extreme environments have already revealed to be a good approach to increase antimicrobials discovery (Arifuzzaman, Khatun, and Rahman 2010; Kay, Pathom-Aree, and Cheeptham 2013; Subramani and Aalbersberg 2013). Although Actinobacteria is a highly prolific bacterial group with about 55% of the existing antimicrobial compounds isolated from the Streptomyces genus and 11% from other genera (Hopwood 2007), in our study none of the isolates belonging to this phyla showed antimicrobial capacity against any of test agents. On the other hand, *Firmicutes* and *Proteobacteria*, revealed the most promising antimicrobial properties with genus *Bacillus* and

Pseudomonas, respectively. Some studies reported antimicrobial activity of Pseudomonas species (Cardozo et al. 2013) and some already isolated the substance responsible for the activity, namely zafrin [4b-methyl-5, 6, 7, 8 tetrahydro-1 (4b-H)- phenanthrenone (Uzair et al. 2008). *Bacillus* species are well-known producers of a wide range of antimicrobial compounds, including peptides, lantibiotics and other bacteriocins (Abriouel et al. 2011). Also, antimicrobials produced by members of the genus Bacillus are more active against Gram-positive bacteria (Slepecky and Hemphill 1986). Further analysis should be performed in order to isolate and characterize the antimicrobials produced by these strains, contributing to the discovery of novel antibiotics. In addition, and considering the potential already observed in other studies and reviewed by Gosh and co-workers (2017), the bioactivity assessment should go beyond antimicrobial activity and also encompass activities such as the antitumoral.

Overall this study contributes for the knowledge of Portuguese cultivable cave bacterial diversity, being to our knowledge, the first being performed on the Algarve karstic region. Furthermore, this work also reinforces the idea that prospection of new antimicrobial compounds in subterranean environments is a promising approach in order to fight spreading of antimicrobial resistance problem.

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III - Final Remarks

1- Final Remarks

On a global perspective, the work carried out in this dissertation intended to contribute for the generalised effort of searching new potential sources of antibiotics. By developing this work, we were able to contribute for two specific aims: i) perform the first prospection of bacteria with antimicrobial activity in Algarve's karstic caves and ii) contribute to the filling the existent knowledge gap concerning Portuguese caves' microbial diversity, namely focusing on cultivable bacteria.

In order to achieve the proposed goals, three Algarve karstic caves were sampled namely Vale do Telheiro, Ibne Ammar and Senhora caves. This choice resulted from the fact that caves are oligotrophic environments with no light and high humidity levels and that in the selected caves had never been prospected. In fact, the choice of the sampling sites reflected a current trend for prospecting new bacteria and possibly new compounds in extreme habitats (Axenov-Gribanov et al. 2016; Selama et al.2014). The bacterial isolates from the caves were obtained using common microbiological media and, as a result from the antimicrobial screening, we observed that 53% of the bacterial isolates were capable of inhibiting the growth of at least one test agent. This evidence contributes for the reinforcement of the idea that caves have a great potential for harbouring many bacterial species able to produce antimicrobial compounds (Axenov-Gribanov et al. 2016; Jiang et al. 2015; Herold et al. 2005). The strains with activity belonged to the genus *Viridibacillus, Psychrobacillus, Serratia, Pseudomonas and and Bacillus,* with the latter encompassing the isolates with the most tests agents, both Gram positive and Gram negative. It would be interesting to further assess the bioactivity of the compounds produced by these bacteria, both towards other pathogenic bacteria as test agents, and also their potential as antitumoral compounds. As shown by Selama et al. (2014) bacteria strains from extreme habitats may present both of these bioactivities.

From a bacteria diversity point of view, our strategy provided the isolation of 110 bacterial isolates from the three caves. After phylogenetical analysis, the bacterial isolates were affiliated to three bacterial phyla: Firmicutes, Proteobacteria and Actinobacteria. In general, the cultivable bacteria diversity was similar between the caves, being grouped in a total of 19 genera. These

results might be the consequence of the restricted number of culture media employed in this study. Also, the use of other media, or supplements, can be a valuable addition in further studies in order to obtain other bacterial groups. Furthermore, a dereplication should be applied to eliminate identical strains. Other strategy that could favour our findings can be to simulate in the laboratory the subterranean environment: absence of natural light, controlled temperatures from 10 to 12 Celsius degrees and air humidity of 98-100% during the incubation period. Furthermore, longer incubation periods, could also be beneficial for the isolation of slow-growth bacterial strains.

Considering the results obtained in this dissertation, further studies should be performed, including testing the bacterial supernatants of the most active strains and optimizing the culture conditions (temperature, pH, broth media) to increase the production of the antimicrobial compounds responsible for the activity observed in our antagonistic tests. Furthermore, chemical analysis of the extracts should be performed in order to isolate the antimicrobial compound and elucidate about his structure.

Overall, subterranean environments should be further studied, firstly to evaluate the diversity of microorganisms that occur there, secondly to understand their role in caves' ecosystem and lastly to assess and extract bioactive compounds produced by these microorganisms in order to suppress the current demand for these compounds, namely antibiotics.

2- References

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